Carbohydrate Exudation from Pea Seeds: Effect of Cultivar, Seed Age, Seed Color, and Temperature

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ABSTRACT

Exudates from pea (*Pisum sativum*) cultivars Miragreen and Alaska were aseptically collected from sterile seeds and analyzed by the anthrone method for total carbohydrate. The preponderance of carbohydrate was exuded during the first 18 hours of incubation at 22 or 30 C, but at 10 C significant carbohydrate exudation persisted for about 48 hours. Total carbohydrate exuded during the first 48 hours of seed germination ranged from 185 to 7,119 μg glucose equivalents per seed depending on cultivar, seed age, seed color, rate of imbibition of water, and incubation temperature. Total carbohydrate exuded by Alaska seeds was not affected by temperature. Eight-year-old Miragreen seeds imbibed water faster and exuded several times more carbohydrate during the first 48 hours of germination than did Miragreen seeds which were less than one year old, and yellow Miragreen seeds exuded significantly more carbohydrate than did green Miragreen seeds of a similar age. Conditions which favored high amounts of nutrient exudation generally corresponded with conditions which have favored high levels of pathogen spore germination around seeds and high amounts of seedling decay, suggesting a direct causal relationship between amounts of carbohydrate released from seeds and disease incidence.

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Nutrients exuded during seed germination diffuse into surrounding soil where they stimulate microbial activity; this is known as the spermosphere effect (24, 28). Most of the carbohydrates exuded by peas (12) and beans (22) are simple sugars such as glucose, sucrose, fructose, and maltose. These sugars are capable of stimulating spore germination and germ tube growth of seed-rotting fungi (8, 22). Incidence of seed rot was directly correlated with the quantity of carbohydrate exuded in vitro by soybeans (10), beans (20), and peas (17). However, the relationship between in vitro nutrient exudation and pathogen activity in spermosphere soil has not been critically examined. Direct quantitative measurement of seed exudates in natural soils has been impossible because sugars and amino acids are quickly taken up and metabolized by indigenous soil microflora (1, 2, 11, 13). However, exudation has been measured in vitro from seeds germinating immersed in water (12, 17, 19), on moist filter paper (20, 21, 26), on cheese cloth (9), and in sterile moist sand (21, 23). This investigation sought to refine in vitro aseptic techniques for seed exudate collection, and to examine the relation between seed exudation and the magnitude of the spermosphere effect (24, 27).

Pea (*Pisum sativum* L.) cultivars Miragreen and Alaska were selected for this study for the following reasons: (i) the magnitude of the spermosphere effect with *Fusarium solani* f. sp. *pisi* chlamydospore germination used as an index had been determined for the wrinkled-seeded Miragreen and the smooth-seeded Alaska cultivars (24), but amounts of exudates had not been directly measured; (ii) the dramatic decrease in the spermosphere effect about Miragreen pea seeds following immersion for 48 hours in water prior to planting (24) merited explanation; (iii) the effect of temperature on carbohydrate exudation from these seeds had not been examined, or was inconclusive (23); and (iv) the effect of seed age (26) and loss of green color (6, 15) on seed exudation had not been investigated under sterile conditions.

MATERIALS AND METHODS.—*Source and treatment of seeds.*—Pea seeds were purchased from Ferry-Morse Seed Co., Mountain View, Calif. All seeds were less than 1 year old when used, except in one experiment in which 8-year-old seeds also were planted. Seeds were selected which weighed 220-240 mg (fresh weight) per seed, and those with spotted or cracked seed coats were discarded. Miragreen seeds were separated into yellow, yellow-green, and green lots. All seeds were surface-sterilized for 30 minutes in 0.5% sodium hypochlorite containing 1 ml of Tween-20 (polyoxyethylene sorbitan monolaurate) per liter, and rinsed for 5 minutes in sterile distilled water. Seeds with defective seed coats began to swell during this pregerminative treatment, and were discarded. Seed swelling was the visual criterion used to determine the time required for complete water imbibition by seeds in moist glass beads or in sterile distilled water.

Collection of exudates.—Two types of sterile leaching systems were used to collect exudates during the first 2-4 days of seed germination at 10, 22, and 30 C.

One system (Fig. 1-A) consisted of a separatory funnel connected with Tygon tubing to a Pyrex glass cylinder (25-mm-diameter × 90 mm) with a rubber stopper at each end, containing 20 g of 1-mm-diameter glass beads, and equipped with an air vent plugged with cotton, and a 7-mm-diameter drainage outlet. Following sterilization of this apparatus, sterile distilled water was poured aseptically into the separatory funnel, a single surface-sterilized pea seed was placed aseptically within the glass bead matrix, and the glass cylinder was covered with aluminum foil to exclude light. Water was then

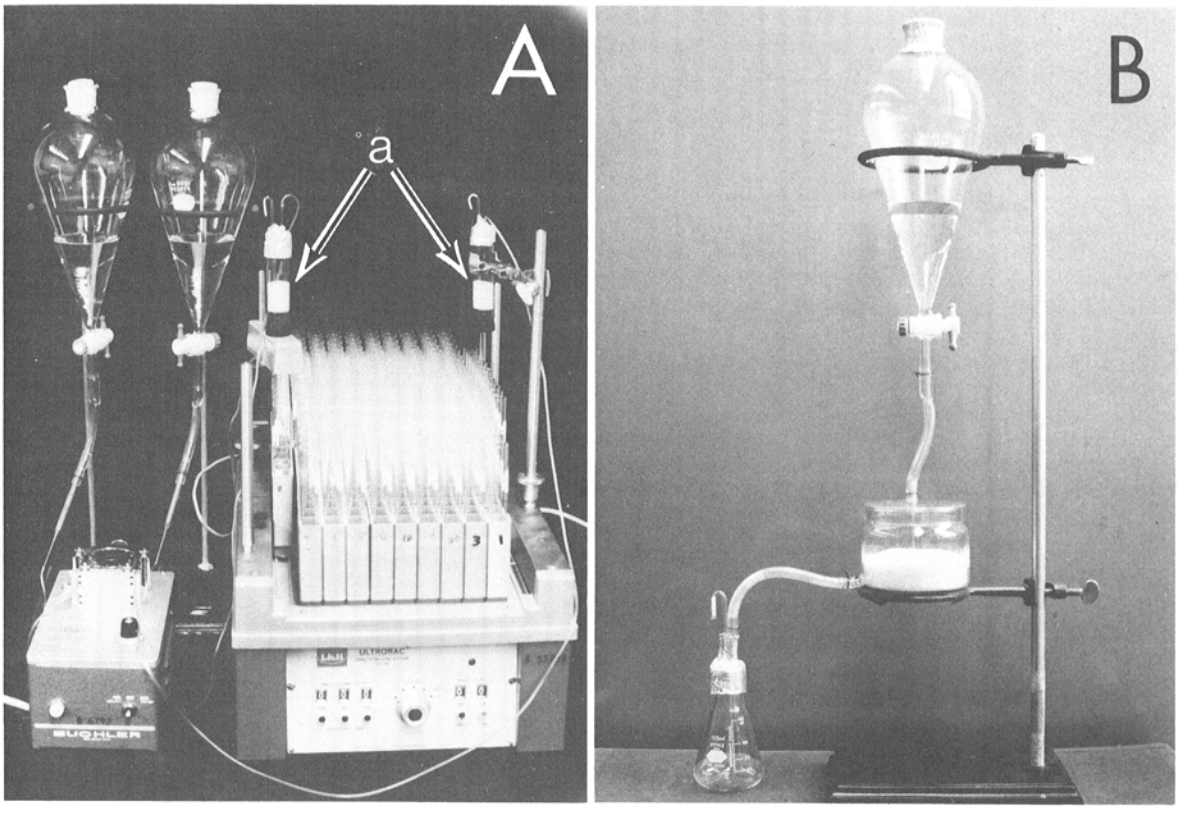

Fig. 1-(A, B). Sterile leaching systems for collecting exudates from surface-sterilized pea seeds during 4 days of germination in glass beads. **A)** Peristaltic pump delivered 10 ml of water per hour to glass beads (a) in which a single seed was germinating; leachings were collected each hour on a fraction collector. **B)** Exudates from 10 seeds germinating in glass beads in a modified petri dish were collected in water washes every 8 hours.

percolated through the glass bead matrix at a rate of 10 ml/hour using a peristaltic pump. A fraction collector was utilized for collecting the leaching water at hourly intervals in test tubes containing 10 ml of 95% ethanol to prevent possible utilization of exudates by contaminating microorganisms in the collection tubes.

The other leaching apparatus (Fig. 1-B) consisted of a modified petri dish (100-mm-diameter × 80 mm) containing 300 g of 1-mm-diameter glass beads, which was connected to a separatory funnel containing sterile distilled water positioned above, and to a collection flask below. Surface-sterilized pea seeds were individually soaked for 8 hours in 5 ml of sterile distilled water, and were placed in groups of 10 seeds each according to the apparent amount of time (5, 6, or 8 hours) required to complete swelling. All soak waters were retained for carbohydrate analysis. Seeds which had completed swelling after 5, 6, or 8 hours were transferred after 8 hours of immersion to a leaching system for exudate collection. Seeds which had not completed swelling after 8 hours were left in water an additional 4 hours until swelling was visually complete before transfer from soak water to a leaching system. Exudates were collected from leaching systems every 8 hours by twice flooding the glass beads with 35 ml of sterile distilled water, and allowing the leachings to drain into the collection flask.

In one experiment, surface-sterilized seeds were individually immersed in 5 ml of sterile distilled water for 24 or 48 hours, and then were grouped according to imbibition rate. Ten seeds of a comparable imbibition rate were aseptically placed within the glass bead bed in each petri dish (Fig. 1-B). Exudates were collected after the 24- or 48-hour immersion, and again 24 hours after planting.

Analysis of exudates.—Leachings from both systems were tested for sterility on potato-dextrose agar, and contaminated experiments were discarded. Cellular debris was removed from the leachings by filtration through a 0.22-μm (pore diameter) Millipore filter. Leachings from petri dish systems containing 10 seeds were condensed to dryness at 40 C with a Rotavapor high-vacuum evaporator, and redissolved in 10-25 ml of distilled water. Leachings were collected hourly from individual seeds germinating in glass cylinders and condensed to dryness at 40 C using a manifold with pasteur pipets directing a filtered air stream into each test tube containing leachings. Exudates were then redissolved in 1 ml of distilled water, to which was added 9 ml of anthrone reagent (18). After 10 minutes in a boiling water bath, optical density at 600 nm was measured with a Bausch and Lomb Spectronic 20 spectrophotometer, and total carbohydrate concentrations were determined using glucose as a standard. Experiments in which exudates were collected hourly from individual seeds were repeated

five times; experiments with 10 seeds per petri dish were repeated once. Significant differences between means were determined ($P = 0.05$) by the use of Duncan's multiple range test (7).

The quantitative determination of carbohydrate exuded every hour from individual pea seeds germinating in sterile glass beads represented a refinement of previous methods (9, 12, 17, 19, 20, 21, 23, 26), and had the following advantages: (i) variability among seeds could be determined, eliminating the possibility that average amounts of exudation might be distorted by a few unusually leaky seeds (19); (ii) the probability of contamination was much less when a single surface-sterilized seed was transferred to a sterile environment than when many seeds were involved, and contaminated or nonviable replicates were easily discarded without nullifying the entire experiment; and (iii) a glass bead environment more nearly represented a natural soil environment, whereas seed exudation has usually been studied when seeds germinated submerged in water.

RESULTS.—The greater part of total carbohydrate measured during 96 hours was exuded by each cultivar during the first 18 hours of incubation at 22 and 30 C, but significant exudation persisted for about 48 hours at 10 C (Fig. 2). Individual seeds of both cultivars consistently revealed two peak exudation periods at all three temperatures (Fig. 2), although the second Miragreen peak at 10 C was obscured by the different times (14 to 37 hours) at which exudation from individual seeds was most intense.

Effect of temperature.—Miragreen peas exuded more carbohydrate in 96 hours when incubated at 10 C than at 22 or 30 C (Table 1), although the difference between 10 and 30 C was not statistically significant. Total exudation from Alaska peas was not significantly affected by temperature (Table 1), even though the pattern of exudation was temperature-dependent.

Effect of cultivar.—Rate of carbohydrate exudation from Miragreen seeds was significantly greater than from Alaska seeds during the first 18 hours at 10 C, during the first 4 hours at 22 C, and during the first 7 hours at 30 C. Carbohydrate exudation from Miragreen seeds from 18 to 48 hours was significantly greater than from Alaska seeds only at 10 C, and differences in rates of exudation were not significantly different from 48 to 96 hours at any temperature.

Effect of seed age.—Eight-year-old Miragreen seeds swelled more quickly, and exuded up to 10 times more carbohydrate during the first 2 days of germination at 22 C than did seeds which were less than one year old (Table 2).

Relation to seed color.—Total carbohydrate exudation during 2 days of germination at 22 C was considerably greater from yellow than from green Miragreen seeds of a comparable age (Table 2), and exudation from yellow seeds subsided more slowly than that from green seeds (Tables 2 and 3). Both the amount and the rate of exudation from yellow-green seeds were intermediate between green and yellow seeds (Tables 2 and 3).

Relation to imbibition rate.—More time was required to complete seed swelling at low (10 C) than at high (30 C) incubation temperatures (Table 1). Seeds which imbibed water rapidly exuded more carbohydrate than seeds of a comparable age and color which imbibed more slowly at

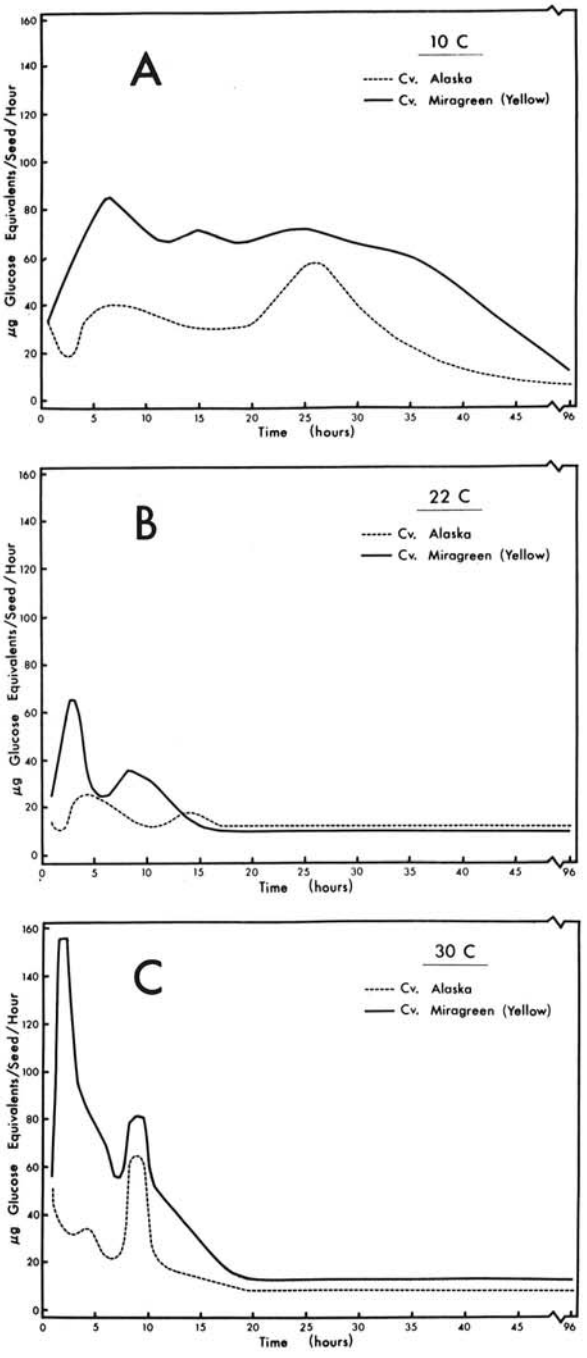

Fig. 2-(A to C). Average amount of carbohydrate exuded from six Miragreen and six Alaska pea seeds at hourly intervals during the first 4 days of seed germination in glass beads at **A)** 10 C. **B)** 22 C. **C)** 30 C.

22 C (Tables 2 and 3). Seeds submerged in water swelled more rapidly and exuded more carbohydrate than seeds incubated in moist glass beads.

Effect of immersion of seeds in water.—Incidence of pea seed and seedling rot in growth chamber and field tests was reduced by a 48-hour preplanting soak in water at 22 C (25). Miragreen seeds exuded larger amounts of

TABLE 1. Effect of temperature and cultivar on water imbibition and carbohydrate exudation during 4 days of pea seed germination in sterile glass beads

Temperature (C)	Cultivar	Apparent imbibition time[x] (hours)	Carbohydrate exudation (μg carbohydrate/seed/hour[y]) during Initial 18 hours	Subsequent 78 hours	Total per seed (96 hours)
10	Alaska	15 (11-17)	32.9 ab[z]	13.5 a	1,642 a
	Miragreen (Yellow)	23 (15-32)	67.9 c	27.6 b	3,378 b
22	Alaska	13 (11-16)	16.4 a	11.7 a	1,206 a
	Miragreen (Yellow)	8 (6-14)	27.7 ab	9.7 a	1,253 a
30	Alaska	8 (8-10)	28.6 ab	8.4 a	1,168 a
	Miragreen (Yellow)	7 (6-12)	60.3 bc	13.3 a	2,123 ab

[x]Mean and range of time required for complete swelling of seeds (15 per treatment).
[y]Glucose equivalents, mean of six seeds.
[z]Means in each column followed by the same letter do not differ significantly ($P = 0.05$) by Duncan's multiple range test.

TABLE 2. Relation of cultivar, seed color, age, and imbibition rate to carbohydrate exudation at 22 C. Sterile pea seeds were individually immersed for 8 hours in sterile distilled water, or until imbibition was completed, and were then placed within the glass beads of a petri dish leaching system. Exudates were collected during the imbibition period and during the remainder of 2 days in sterile glass beads

Cultivar	Seed color	Seed age (years)	Apparent imbibition time[x] (hours)	Carbohydrate exudation (μg carbohydrate[y]/seed/hour) Day 1	Day 2	Total carbohydrate/seed (48 hours)
Miragreen	Yellow	8	5	268.8 a[z]	27.9 a	7,119 a
	Yellow	<1	6	51.2 b	5.8 b	1,368 b
	Yellow	<1	8	29.9 c	3.5 bc	802 c
Miragreen	Yellow-green	<1	8	28.3 c	1.0 cd	705 c
Miragreen	Green	8	6	131.8 d	12.1 e	3,452 d
	Green	<1	8	12.3 ef	0.5 d	308 ef
	Green	<1	12	7.3 e	0.5 d	185 e
Alaska	Green	<1	8	18.1 f	0.9 d	456 f

[x]Time required for seeds to become completely swollen during immersion in water.
[y]Glucose equivalents, mean of 10 seeds per treatment, two replications.
[z]Means in each column followed by the same letter do not differ significantly ($P = 0.05$) by Duncan's multiple range test.

carbohydrate during the 48 hours of immersion in water than during a subsequent 24-hour incubation in a glass bead leaching system (Table 3). Yellow seeds exuded several times more carbohydrate than did green seeds during immersion and in the subsequent 24-hour incubation in glass beads. As times of immersion of yellow Miragreen seeds were increased from 0 to 48 hours prior to transfer to a glass bead leaching system, total carbohydrate exuded during the entire 48-hour period increased in direct proportion to the amount of time seeds were immersed in water. For 0, 8, 24, and 48 hours of immersion in water followed by 48, 40, 24, and 0 hours in glass beads, total carbohydrate (glucose equivalents) exuded per seed was 828, 1,085, 1,285, and 2,065 μg, respectively.

DISCUSSION.—Imbibition of water by pea seeds in glass beads was visually complete in 6-16 hours at 22 and 30 C, but required 11-32 hours at 10 C (Table 1). Reports (8, 12, 26) that pea seed exudation subsides following completion of water imbibition were confirmed. Carbohydrate was exuded in large amounts for approximately 24 hours longer at 10 C than at 22 or 30 C (Fig. 2).

The simultaneous uptake of water and exudation of cell solutes by germinating pea seeds apparently occurs over the entire seed surface; however, the micropyle is thought to be the most active site (14, 24). Sugars and amino acids are exuded primarily from the symplast of the cotyledons (12, 26), though the seed coat and superficial residues may also contribute small amounts of solutes.

Two mechanisms of exudation have been proposed. Larson (12) observed that submerged pea seeds imbibed water and exuded solutes more readily following removal of the seed coat; he proposed that rapid imbibition disrupts membrane organization, resulting in the leakage of cell contents. Simon and Raja Harun (26) hypothesized that cell membranes lose their integrity as seeds dry at maturity, and thus solutes leak out of cells during imbibition while membrane integrity is being re-established. Electron micrographs of pea cotyledons revealed a fragmentation and gradual breakdown of

membranes during maturation (3), and their subsequent redevelopment during germination (4). Larson's (12) hypothesis would predict membrane damage and subsequent exudation to be less at 10 C than at 22 or 30 C because of a slower rate of imbibition at lower temperatures. On the contrary, carbohydrate exudation was greater at 10 C than at 22 or 30 C (Table 1). Moreover, imbibition at 10 C was more rapid for Alaska (15 hours) than for Miragreen (23 hours) peas, yet Alaska peas exuded less carbohydrate than Miragreen peas (Table 1). Our data support the hypothesis of Simon and Raja Harun (26).

Individual seeds of both cultivars consistently revealed two peak exudation periods at all three temperatures (Fig. 2). Simon and Raja Harun (26) also observed a biomodal curve of carbohydrate exudation. Seed metabolism was probably quite low during the times coinciding with the first exudation peak, since imbibition just would have begun; however, metabolic processes are known to increase rapidly during times coinciding with the second exudation peak (15). The second peak of carbohydrate exudation may reflect increasing catabolic activity, associated with restoration of membrane integrity. Indeed, exudation subsided more quickly as temperature was increased from 10 to 30 C (Fig. 2), as would be expected if metabolic activity was involved. Since the sugar content of pea seeds increases during the 1st week of germination (4), the reduction in rate of carbohydrate exudation during the 3rd and 4th days of germination (Fig. 2) may reflect restoration of membrane integrity, rather than depletion of carbohydrate reserves.

Temperature affected the pattern of exudation (Fig. 2), but did not significantly affect total carbohydrate exuded from smooth-seeded Alaska peas with (Table 1) or without (23) seed coats. However, both quantity and composition of carbohydrate exuded by Alaska peas lacking seed coats was different than from seeds with intact testae (12). Wrinkled-seeded peas exuded more electrolytes at 10 C than at 20 C (19). Similarly, the first 18 hours of in vitro carbohydrate exudation from wrinkled-seeded Miragreen peas in our experiment was greater at 10 and 30 C than at 22 C (Table 1). The inverse relation between temperature and spermosphere size in soil at 50% moisture (24) may be due to greater microbial competition for exudates diffusing away from seeds at 30 C than at 22 or 10 C (2).

Maturation of pea seeds during moist, hot, sunny weather causes many green peas to turn yellow (15) due to chlorophyll loss (5), a process known as bleaching or "blonding" (6, 15, 29). Green and yellow peas did not differ in quantity or composition of carbohydrates (15); however, leakage of sugars, amino acids, and carboxylic acids was greater from yellow than from green seeds germinating in nonsterile water (15) or in sterile glass bead environments (Tables 2 and 3). Further, exudation from yellow and green seeds increased with maturation (26) and age (Table 2) (15). Loss of moisture as seeds mature (16) may accompany loss in membrane structure (3). Stressful maturation conditions, such as those in which "blonding" of peas occurs, may alter membrane systems (3) and permit excessive exudation during seed germination (16).

Miragreen seeds exuded more carbohydrate than Alaska seeds during the first 12-20 hours of seed germination at 10, 22, or 30 C (Fig. 2). Similarly, the magnitude of the spermosphere effect as measured by *F. solani* f. sp. *pisi* chlamydospore germination (24) was greater with Miragreen than with Alaska peas, indicating a causal relation between nutrient exudation and spore germination in the spermosphere. Moreover, seed exudation subsided after 24-48 hours of immersion in water at 22 C (Table 3); consequently, when soaked seeds were planted in soil, the spermosphere effect was much smaller than when seeds were not soaked (24). Thus, the amount of carbohydrate exuded in vitro was directly related to the magnitude of the spermosphere effect in soil at 10, 22, or 30 C, and the higher the magnitude of the spermosphere effect, the greater the incidence of pea seed and seedling rot (24, 25).

TABLE 3. Relation of seed color and water imbibition rate of Miragreen peas to carbohydrate exudation during 24 or 48 hours of immersion in sterile distilled water at 22 C, and during a subsequent 24 hours at 22 C in sterile glass beads

Seed color	Apparent imbibition time (hours)[x]	Carbohydrate exudation (μg carbohydrate[y]/seed/hour) Immersion time (hours) 24	48	Glass beads
Yellow	6	51.2 a[z]	...	4.7 a
	6	...	55.8 a	1.1 bc
Yellow	8	55.8 a	...	5.4 a
	8	...	30.2 b	2.2 b
Yellow-green	8	36.9 b	...	2.5 b
	8	...	22.3 b	0.6 c
Green	8	14.2 c	...	0.6 c
	8	...	8.8 c	0.7 c
Green	12	8.3 d	...	0.8 c
	12	...	5.1 c	0.4 c

[x]Time required for seeds to become completely swollen during immersion in water.

[y]Glucose equivalents, mean of 10 seeds per treatment, two replications.

[z]Means in each column followed by the same letter do not differ significantly ($P = 0.05$) by Duncan's multiple range test.

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